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TITLE: COMPARATIVE INFECTIVITY DETERMINATIONS OF DENGUE VIRUS  
VACCINE CANDIDATES IN RHESUS MONKEYS, MOSQUITOES, AND  
CELL CULTURES

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## FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

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## TABLE OF CONTENTS

	Page
1. Introduction.....	1
2. Dengue 1 vaccines.....	1
3. Table 1.....	3
4. Dengue 4 vaccines.....	4
5. Table II.....	5
6. Dengue 2 vaccines (S16803 STRAIN).....	6
7. Table III.....	7
8. Table IV.....	8
9. Dengue 2 (S16681 STRAIN).....	9
10. Table V.....	10
11. Dengue 2 and Dengue 3 vaccines.....	11
12. Table VI.....	12
13. Dengue 3 and Dengue 4 vaccines.....	13
14. Table VII.....	14
15. Conclusions.....	15
16. References.....	16

**SHORT INTRODUCTION FOR THE FINAL REPORT****Introduction:**

Attenuation of virulent viruses are widely used for the development of viral vaccines through the years. These vaccines are made by serial passages of virus in cells from non-natural hosts. The first intents for the development of Dengue virus vaccines was made by serial intracerebral passages of virus in infant of newly-weaned mice (1-5). Primary canine kidney (PCK) cell culture are also used for the attenuation of wild Dengue virus strains. Studies using these attenuated virus strains have been recently published (6).

The objectives of this contract were to determine the antibody response of rhesus macaques inoculated with different attenuated dengue virus preparations. Vaccine immunogenecity was determined by the plaque reduction neutralization test (PRNT,6). The PRNT titer of each serum sample was determined as described elsewhere (6). The following candidate vaccines were tested: Dengue 1:45AZ5, PDK10,20 and 27; Dengue 4:PDK6, PDK10, PDK15, PDK20; Dengue 2 S16803' PDK10, 20, 30 and 50; Dengue 2 S16681; PDK11, PDK40, PDK53; Dengue 3 CH53489,PDK10,PDK20,PDK30; Dengue 3 TVP2342; #1339; Dengue 4 PRHTVP-360; TVP1975; Results obtained are shown below (see BODY).

**BODY:** Completed efforts and the results obtained, from year 01 are as follows:

**Evaluation of Dengue -1 Vaccines:**

On October 16, sixteen rhesus monkeys were sent from Caribbean Primate Center (Sábana Seca) to the Animal Resources Facilities at the Medical Sciences Campus. The monkeys were bled on October 16 and 30 and the sera were stored at -20°C. The Plaque Reduction Neutralization test (PRNT) and the hemagglutination inhibition (HI) test were performed using these serum samples (Pre vaccination bleeds).

The PRNT was performed using LLC-MK2 cells (sent by Dr. Goro Kuno CDC, San Juan Laboratories) grown in multiwell plates. The four dengue virus serotypes were used in these tests. Dengue viruses used were DEN 1 (Hawaii) Rosen TeT3H; DEN 2 (New Guinea "C") Smp 24 (MK<sub>3</sub> H<sub>3</sub>; DEN 3 (Philippine) (H87) Rosen Tc (T<sub>2</sub>) H<sub>1</sub> and DEN4 (Philippine H<sub>241</sub>). T5H<sub>1</sub>. All viruses were previously grown in mosquito cells, (TRA 284 HT AAL C6/36 [HT]).

Vaccines and the other materials were received from Dr. Kenneth Eckels (Walter Reed Institute of Research) on 28/12/89 at 3:00 P.M.

Vaccines received were: 45AZ5 (I-82); DK10; DK20; and DK27.

On December 11, 1989 groups of four monkeys were inoculated s.c. with 0.5 ml of candidate vaccines. The titration of four vaccine candidates were made on the same day of inoculation using LLC-MK2 cells.

Vaccine titers obtained were as follows:

<u>Vaccine</u>	<u>Titer obtained</u> <u>(Pfu/ml)</u>	<u>WRAIR Titer*</u>
45AZ5	$5.9 \times 10^3$	$5.3 \times 10^4$
DK10	$9.5 \times 10^3$	$6.3 \times 10^3$
DK20	$9.5 \times 10^3$	$8 \times 10^3$
DK27	570	$1.2 \times 10^4$

\*Titer obtained at WRAIR was reported by phone on February 27, 1990. WRAIR titers are expressed in Pfu/0.5 ml.

As stated above, all animals were vaccinated with 0.5 ml of above vaccines, with the exception of DK-1 (that was diluted 1:10 before inoculation). Diluent used was sent by WRAIR. Vaccine doses given s.c. to the animals are shown in Table 1.

The LLC-MK2 cells sent by Walter Reed Laboratories grew very slowly and were not viable for PRNT assays.

After vaccination, animals were bled for 10 consecutive days. As agreed, these samples were sent to WRAIR for viremia studies. On days 30 and 60 post vaccination (January 11, and February 9, 1990) animals were bled and PRNTs against Dengue-1 were performed. Results of the 30d and 60d bleed are presented in Table 1.

TABLE 1

ANTIBODY RESPONSE OF MONKEYS INOCULATED WITH DIFFERENT DEN-1  
VACCINE CANDIDATES

Monkey No.	Vaccine	Vaccine* Inoculum	Antibody Titers	
			day 30	day 60
J6	I-82	$2.95 \times 10^3$	110	210
M76			210	370
M99			320	43
M142			150	145
J4	DK-10	475	60	280
J7			37	120
M91			105	150
M146			100	63
J1	DK-20	$4.75 \times 10^3$	50	16
J2			50	130
M79			<10	260
M139			<10	140
J3	DK-27	285	<10	16
J5			<10	25
M72			<10	21
M75			<10	<10

\*Pfu/0.5 ml

**Evaluation of Dengue-4 vaccines:**

On March 23, sixteen rhesus monkeys were sent from Caribbean Primate Center (Sábana Seca) to the Animal Resources Facilities at the Medical Sciences Campus. The monkeys were bled on March 23 and April 6, and the sera were stored at -20°C. The Plaque Reduction Neutralization test (PRNT) and the hemagglutination inhibition (HI) test were performed using these serum samples (Pre vaccination bleeds).

The PRNT was performed using LLC-MK2 cells (sent by Dr. Goro Kuno, CDC, San Juan Laboratories) grown in multiwell plates as described above (see evaluation of dengue-1 vaccines).

Vaccines and the other materials were received from Dr. Kenneth Eckels (Walter Reed Institute of Research) on 29/3/90 at 3:00 P.M.

Dengue 4 vaccines (Carib 341750) received were: PDK-6, PDK-10, PDK-15 and PDK-20.

On March 23, 1990 groups of four monkeys were inoculated s.c. with 0.5 ml of candidate vaccines. The titration of the four vaccine candidates were made on the same day of inoculation using LLC-MK2 cells sent by Dr. G. Kuno (CDC) were compared to those obtained with LLC-MK2 cells sent by Dr. Ken Eckels (WRAIR).

Vaccine titers obtained were as follows:

<u>Vaccine</u>	<u>LKLC-MK2 (CDC)</u> <u>(Pfu/ml)</u>	<u>LLC-MK2 (WRAIR)</u> <u>(Pfu/ml)</u>
PDK 6	$2.8 \times 10^5$	$3.3 \times 10^5$
PDK 10	$4.3 \times 10^5$	$5.7 \times 10^5$
PKD 15	$1.8 \times 10^5$	$1.1 \times 10^5$
PDK 20	$1.8 \times 10^5$	$1.1 \times 10^5$

Therefore, the same titers were obtained using LLCMK2 cells received from either CDC or WRAIR.

As stated above, all animals were vaccinated with 0.5 ml of 1:10 dilution of the above vaccines, with the exception of PDK-20 which was used undiluted. Diluent used was sent by WRAIR.

After vaccination, animals were bled for 10 consecutive days. As agreed, these samples were sent to WRAIR for viremia studies. On days 30 and 60 post vaccination (May 23, and June 22, 1990) animals were bled and PRNTs against Dengue-4 were performed.

Results of these tests are presented in Table II.



TABLE II

**ANTIBODY RESPONSE OF MONKEYS INOCULATED WITH DIFFERENT DEN-4  
(Carib 341750) VACCINE CANDIDATES**

Monkey No.	Vaccine	Vaccine Innoculum Pfu/.5ml	Antibody Titers	
			day 30	day 60
	PDK6	$1.4 \times 10^4$		
N31			<10	90
N71			<10	300
N69			28	370
N27			105	370
	PDK10	$2.5 \times 10^4$		
N45			<10	15
N49			220	400
N51			<10	<10
N70			<10	<10
	PDK15	$9 \times 10^3$		
N28			<10	<10
029			43	<10
072			27	40
074			<10	<10
	PDK20	$9 \times 10^4$		
N22			<10	<10
N25			35	29
N48			80	135
N52			<10	<10

## Evaluation of Dengue-2 vaccines:

### I. DEN-2 S16803 STRAIN

On September 4, 1990, sixteen rhesus monkeys were sent from the Caribbean Primate Center (Sábana Seca) to the Animal Resources Facilities at the Medical Sciences Campus. the monkeys were bled on September 4, and on September 17, and the sera were stored at -20°C. The Plaque Reduction Neutralization test (PRNT), and the hemagglutination inhibition (HI) tests were performed using serum samples (Pre-vaccination bleds).

The PRNT was performed using LLC-MK2 cells (sent by Dr. Goro Kuno, CDC, San Juan Laboratories) grown in multiwell plates as described before (see Second Semi-Annual Report, August 31, 1990).

Vaccines and the other materials were received on 10/4/90 from Dr. Kenneth Eckels (Walter Reed Army Institute of Research).

The following vaccines of dengue 2 were received: PDK-10, PDK-20, PDK-30 and PDK-50.

On October 15, 1990, groups of four monkeys were inoculated s.c. with 0.5 ml of each candidate vaccine. The titration of the four vaccine candidates were made on the same day of inoculation using LLC-MK2 cells.

Vaccine titers obtained were as follows:

<u>Vaccine</u>	<u>LLC-MK2</u> <u>(Pfu/ml)</u>
PDK 10	$7.65 \times 10^5$
PDK 20	$4.35 \times 10^5$
PDK 30	$4.20 \times 10^5$
PDK 50	$1.16 \times 10^6$

As stated above, all animals were vaccinated with 0.5 ml of above vaccines, with the exception of PDK-50 (that was diluted 1:7 before inoculation, as instructed by Dr. Eckels). Diluent used was sent by WRAIR.

After vaccination, animals were bled for 10 consecutive days. As agreed, these samples were sent to WRAIR for viremia studies. On days 30 and 60 post vaccination (November 14, and December 12, 1990) animals were bled and PRNTs against Dengue-2 (NGC strain) and Dengue-2 (S16803), were performed.

Results of these tests are presented in Tables III & IV.

TABLE III

ANTIBODY RESPONSE OF MONKEYS INOCULATED WITH DIFFERENT DEN-2  
(S16803) VACCINE CANDIDATES

Monkey No.	Vaccine	Vaccine Innoculum Pfu/..5ml	N. Antibody Titers*	
			day 30	day 60
	PDK-10	$3.8 \times 10^5$		
R04			110	>640
R09			260	320
R17			>640	>640
R30			640	110
	PDK-20	$2.18 \times 10^5$		
R02			580	>640
R19			600	460
R49			>640	>640
R54			>640	>640
	PDK-30	$2.1 \times 10^5$		
R11			160	350
R18			<10	<10
R21			>640	300
R37			32	140
	PDK-50	$0.83 \times 10^5$		
R05			32	140
R08			39	>640
R15			100	350
R36			>640	>640

\*Neutralizing antibodies titers were obtained by the PRNT test, using the NGC strain of DEN-2.

**TABLE IV**  
**ANTIBODY RESPONSE OF MONKEYS INOCULATED WITH DIFFERENT DEN-2**  
**(S16803) VACCINE CANDIDATES**

Monkey No.	Vaccine	Vaccine Innoculum Pfu/.5ml	N. Antibody Titers*	
			day 30	day 60
	PDK-10	$3.8 \times 10^5$		
R04			340	640
R09			560	400
R17			600	580
R30			500	500
	PDK-20	$2.18 \times 10^5$		
R02			>640	700
R19			>640	>640
R49			>1280	>640
R54			>1280	>1280
	PDK-30	$2.1 \times 10^5$		
R11			>640	160
R18			<10	<10
R21			>640	>640
R37			>640	460
	PDK-50	$.83 \times 10^5$		
R05			64	94
R08			155	>640
R15			160	82
R36			640	>640

\*Virus used for PRNT: Parent S16803 STRAIN.

**II. Dengue-2 S16681 STRAIN**

On February 12, 1991, sixteen rhesus monkeys were sent from the Caribbean Primate Center (Sábana Seca) to the Animal Resources Facilities at the Medical Sciences Campus. The monkeys were bled on February 12, and on February 26, and the sera were stored at  $-20^{\circ}\text{C}$ . The Plaque Reduction Neutralization test (PRNT) and the hemagglutination inhibition (HI) test were performed using these serum samples (Pre vaccination bleeds).

The PRNT was performed using LLC-MK2 cells (sent by Dr. Goro Kuno, CDC, San Juan Laboratories) grown in multiwell plates as described before.

Vaccines and the other materials were received on 4/4/91 from Dr. Kenneth Eckels (Walter Reed Institute of Research).

The following vaccines of dengue 2 (strain S16681) including the parent seed were received: PDK-11, PDK-40 and PDK 53.

On April 22, 1991, groups of four monkeys were inoculated s.c. with 0.5 ml of each candidate vaccine. The titration of the four vaccine candidates were made on the same day of inoculation using LLC-MK2 cells.

Vaccine titers obtained were as follows:

<u>Vaccine</u>	<u>LLC-MK2 (CDC)</u> (Pfu/ml)	<u>Titers reported</u> <u>by WRAIR</u> (Pfu/0.2 ml)
S16681	$3.3 \times 10^7$	$1.8 \times 10^6$
PDK 11	$7.9 \times 10^6$	$4 \times 10^6$
PDK 40	$2.2 \times 10^6$	$2.2 \times 10^5$
PDK 53	$3.3 \times 10^4$	$2.2 \times 10^5$

Shown on the right column are the titers obtained at WRAIR. These titers were different to those obtained in our laboratory, using LLC-MK2 cells received from the local CDC laboratory. Very small plaques were observed with PDK 40 and 53. PDK 11 are the parent seed S16681 had approximately the same plaque size.

As stated above, all animals were vaccinated with 0.5 ml of above vaccines, with the exception of PDK-11 (that was diluted 1:18) and the parent seed (that was diluted 1:18) as previously agreed, before inoculation. Diluent used was sent by WRAIR.

After vaccination, animals were bled for 14 consecutive days. As agreed, these samples were sent to WRAIR for viremia studies. On days 30 and 58 post vaccination (May 22, and June 20, 1991) animals were bled and PRNTs against Dengue-2 (S16681 strain) were performed.

Results of these tests are presented in Table V.

TABLE V

ANTIBODY RESPONSE OF MONKEYS INOCULATED WITH DIFFERENT DEN-2  
(S16681) VACCINE CANDIDATES

Monkey No.	Vaccine	Vaccine Innoculum Pfu/0.5ml	N. day 30	Antibody GMT day 60	Titers* GMT
	S16681	$2 \times 10^6$		1838	$\geq 4608$
R25			1250	4800	
R13			2560	5200	
R50			2300	3500	
H5			1550	>5120	
	PDK11	$2.2 \times 10^5$		1267	$\geq 4104$
J15			800	4100	
R28			1280	2700	
R41			1800	5000	
J16			1400	>5120	
	PDK40	$1.1 \times 10^5$		338	1652
J21			560	2560	
R35			260	2000	
J20			150	470	
J22			600	3100	
	PDK53	$1.1 \times 10^4$		323	1290
R6			640	4300	
R16			330	1120	
R1			270	900	
R38			190	640	

\*Virus used for PRNT: Parent seed S16681 STRAIN  
GMT = Geometric mean titer

**Evaluation of Dengue-2 and Dengue-3 vaccines:**

On August 21, 1991, sixteen rhesus monkeys were sent from the Caribbean Primate Center (Sábana Seca) to the Animal Resources Facilities at the Medical Sciences Campus. The monkeys were bled on September 4, and on September 17, and the sera were stored at -20°C. The Plaque Reduction Neutralization test (PRNT), and the hemagglutination inhibition (HI) tests were performed using these serum samples (Pre-vaccination bleds).

The PRNT was performed using LLC-MK2 cells (sent by Dr. Goro Kuno on 11/14/91 from Dr. Kenneth Eckels (Walter Reed Army Institute of Research)).

The following vaccines were received: DEN 2 (S16681) PDK53, DEN3 (CH53489) parent seed, PDK10, PDK20, PDK30, and DEN4 (1036) parent seed.

On December 2, 1991 groups of three monkeys were inoculated s.c. with 0.5 ml of each candidate vaccine. One monkey was inoculated with 0.9ml of DEN4 1036 (parent seed). The titration of the three vaccine candidates and the parent seeds were made on the same day of inoculation using LLC-MK2 cells.

Vaccine titers obtained were as follows:

<u>STRAINS</u>	<u>Titer Obtained</u> (pfu/ml)	<u>Titer WRAIR</u> (pfu/0.2ml)
DEN2 (16681) PDK53	$3.95 \times 10^4$	$2.2 \times 10^5$
DEN3 parent (CH53489)	$1.6 \times 10^5$	$2.3 \times 10^5$
DEN3 PDK10	$1.3 \times 10^4$	$1.1 \times 10^4$
DEN3 PDK20	$5.9 \times 10^3$	$4.4 \times 10^3$
DEN3 PDK30	$4.7 \times 10^3$	$6.3 \times 10^3$
DEN4 (1036) parent	$2.95 \times 10^6$	$4.2 \times 10^6$

As stated above, all animals were vaccinated with 0.5 ml of above vaccines, with the exception of DEN3 CH53489 (parent seed) that was diluted 1:20 before inoculation, as instructed by Dr. Eckels. Diluent used was sent by WRAIR.

After vaccinations, animals were bled for 10 consecutive days. As agreed, these samples were sent to WRAIR for viremia studies. On days 30 and 60 post vaccination (December 2, 1991 and February 3, 1992) animals were bled and PRNTs against Dengue-2 (S16681), Dengue-3 (CH53489), DEN4 (1036) and DEN4 (341750 CARIB) were performed.

Results of these tests are presented in Table VI.

TABLE VI

ANTIBODY TITERS OF MONKEYS VACCINATED WITH DIFFERENT  
DENGUE VIRUS STRAINS

<u>Monkey No.</u>	<u>Vaccine</u>	<u>Vaccine Innoculum</u> (Pfu/0.5ml)	<u>N. Antibody Titers</u> (day 30 - day 60)	
	(DEN-3) CH53489	$4. \times 10^3$		
M96			320	540
M147			960	540
M172			320	560
	(DEN-3) PDK10	$6.6 \times 10^3$		
M152			160	120
M192			160	58
M213			120	74
	(DEN-3) PDK30	$2.95 \times 10^3$		
M168			74	48
M207			1,010	80
M215			30	16
	(DEN-3) PDK30	$2.35 \times 10^3$		
M156			<10	<10
M166			<10	<10
M171			<10	<10
J11	DEN4 (1036)	$2.7 \times 10^6$ *	33** 430***	250** ~640***
M158	DEN2 (S16681)	$2 \times 10^4$	350	600
M202	PDK53		640	≥640
M214			320	≥640

\* DEN4 inoculum was in 0.9 ml

\*\* N titer against DEN4 (1036)

\*\*\* N titer against DEN4 (341750 CARIB)

Date of this report 2/28/92



**Evaluation of Dengue-3 and Dengue-4 strains:**

On May 5, sixteen rhesus monkeys were sent from Caribbean Primate Center (Sábana Seca) to the Animal Resources Facilities at the Medical Sciences Campus. The monkeys were bled on May 5 and on June 4, and the sera were stored at -20°C. The Plaque Reduction Neutralization test (PRNT), the ELISA IgM and the hemagglutination inhibition (HI) test were performed using these serum samples (Pre vaccination bleeds).

The PRNT was performed using LLC-MK<sub>2</sub> cells (sent by Dr. Goro Kuno CDC, San Juan Laboratories) grown in multiwell plates as described in previous reports.

Dengue strains received were: D-3 TVP-2342, D-3 #1339, D-4 PRH TVP-360, and D-4 TVP 1975.

On June 16, 1992 groups of four monkeys were inoculated s.c. with 0.5 ml of each dengue strain. The titrations of the four dengue strains were made on the same day of inoculation using LLC-MK<sub>2</sub> cells. Titers obtained using LLC-MK<sub>2</sub> cells sent by Dr. G. Kuno (CDC) were compared to those obtained by WRAIR.

Dengue strains titers obtained were as follows:

Strain	LLC-MK2 (CDC) (Pfu/ml.)	LLC-MK2 (WRAIR) (Pfu/0.2ml)
D-3 TVP-2342	$4.5 \times 10^3$	$2.1 \times 10^4$
D-3 #1339	None	$4.8 \times 10^4$
D-4 PRH TVP-360	$8.2 \times 10^4$	$8.8 \times 10^5$
D-4 TVP 1975	$8.0 \times 10^2$	$3.3 \times 10^5$

Note that no titer was obtained by D-3 #1339 strain. This was confirmed by WRAIR at a later date.

All animals were vaccinated with 0.5 ml of above dengue strains. After inoculation, animals were bled for 14 consecutive days. As agreed, these samples were sent to WRAIR for viremia studies. On days 30 and 60 post inoculation (July 16 and August 13, 1992) animals were bled and PRNTs against Dengue-3 H87 (Rosen) and Dengue-4 H241 were performed.

Results of these tests are presented in Table VII.

TABLE VII

ANTIBODY RESPONSE OF MONKEYS INOCULATED WITH DIFFERENT  
DEN-3 & DEN-4 PREPARATIONS

Monkey No.	Virus	Virus Inoculum (pfu/0.5ml)	Antibody Response	
			Day 30	60
	DEN-3 TVP-2342	$2.3 \times 10^3$	36	120
T33			160	280
T40			125	430
T64			40	130
	DEN-3 #1339	NONE DETECTED		
T14			<10	24
T48			23	130
T49			<10	58
V48			<10	15
	DEN-4 PRH-TVP-360	$4.1 \times 10^4$		
T68			150	450
V28			100	320
V31			155	280
V33			49	140
	DEN-4 TVP-1975	$4 \times 10^2$		
S02			112	150
T50			380	230
V23			12	43
V62			25	76

### CONCLUSIONS

- 1) A very weak antibody response was observed in monkeys vaccinated with DEN-1 45AZ5 strains DK20 and DK27. This implies that high "in vitro" passage of this vaccine candidate results in loss of its immunogenicity in rhesus monkeys.
- 2) Loss of immunogenicity was also observed in high passages of the DEN-4 (carib 341750) vaccine candidate.
- 3) All Dengue 2 vaccine candidates (S16803 strain) were immunogenic. Titers obtained against DEN2 S16803 were higher than those obtained with DEN-2 NGC.
- 4) All Dengue 2 vaccine candidates (S16681 strain) induced high antibody titers. "In vitro" passage of this vaccine did not result in loss of its immunogenicity.
- 5) Further studies "in vivo" are required for the evaluation of these vaccine candidates.

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